Phosphoproteins associated with cyclic nucleotide stimulation of ciliary motility in *Paramecium*

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Summary

Permeabilized, MgATP-reactivated cells of *Paramecium* (models) respond to cyclic AMP and cyclic GMP by increasing forward swimming speed. In association with the motile response, cyclic AMP and 8-bromo-cyclic GMP (8-Br-cyclic GMP) stimulated protein phosphorylation. Cyclic AMP addition to permeabilized cells reproducibly stimulated the phosphorylation of 10 proteins, ranging in molecular weight from 15 to 110K (K=10^3 M_r). 8-Br-cyclic GMP, which selectively activates the cyclic GMP-dependent protein kinase of *Paramecium*, stimulated the phosphorylation of a subset of the proteins phosphorylated by cyclic AMP. Ca^{2+} addition caused backward swimming and stimulated the phosphorylation of four substrates, including a 25K target that may also be phosphorylated in response to cyclic nucleotide addition. Ba^{2+} and Sr^{2+} also induced backward swimming, but did not cause detectable phosphorylation.

To identify ciliary targets of cyclic nucleotide-dependent protein kinase activity, permeabilized cells were deciliated following reactivation of motility with Mg-[γ-^{32}P]ATP in the presence or absence of cyclic nucleotide. Soluble proteins of the deciliation supernatant were enriched in 15 cyclic AMP-stimulated phosphoproteins, ranging in molecular weight from 15 to 95K. Most of the ciliary substrates were axonemal and could be released by high salt solution. A 29K protein that copurified in sucrose gradients with the 22 S dynein, and a high molecular weight protein (>300K) in the 19 S region were phosphorylated when cyclic AMP was added to permeabilized, motile cells. These data suggest that regulation of ciliary motility by cyclic AMP may include phosphorylation of dynein-associated proteins.

Key words: *Paramecium*, cyclic AMP, cyclic GMP, Ca^{2+}, dynein, cilia, Triton-permeabilized cells, swimming speed, ciliary reversal, protein phosphorylation.

Introduction

Dynamic regulation of ciliary and eukaryotic flagellar motion is achieved through cyclic AMP or Ca^{2+} functioning as second messengers (Lindemann, 1978; Garbers and Kopf, 1980; Tash and Means, 1983; Satir, 1985; Brokaw, 1987; Bonini *et al.* 1990). Whereas the structure of the ciliary and flagellar axoneme and the role of dynein ATPase in producing sliding motion have been extensively studied (Gibbons, 1981), less is understood of the pathways that modify these interactions to yield a finely controlled, rapidly modulated beat. The unicellular ciliate *Paramecium* is an excellent system in which to explore these regulatory pathways. Because *Paramecium* is amenable to electrophysiological analysis, much is known of the events that couple the membrane response to the motility (Saimi and Kung, 1987). Furthermore, biochemical analysis can be complemented by genetics, with the isolation of mutants behaviorally defective in specific aspects of motility control (Saimi and Kung, 1987).

The swimming behavior of the cell reflects the activity of the many cilia that cover its surface. Motility is modified by regulation of ciliary beat frequency and beat direction in response to various types of environmental stimuli that alter the cell membrane potential. Upon membrane depolarization, intracellular Ca^{2+} rises due to the opening of voltage-dependent Ca^{2+} channels (Eckert, 1972; Saimi and Kung, 1987). Ca^{2+} then acts upon the axoneme to effect backward swimming (Naitoh and Kaneko, 1972). In response to stimuli that induce membrane hyperpolarization, cyclic AMP levels increase

(Bonini *et al.* 1986) and stimulate ciliary activity, causing fast forward swimming of the cell (Hennessey *et al.* 1985; Nakaoka and Ooi, 1985; Bonini and Nelson, 1988).

Cells permeabilized by detergent (models) have been used to investigate modulation of axonemal motility by second messengers and the biochemical targets of second messengers within the axoneme (Naitoh and Kaneko, 1972; Gibbons and Gibbons, 1972; Lindemann, 1978). Such cells are immotile, but the cilia or flagella can be reactivated to beat and the cells to swim by the addition of MgATP, the substrate for dynein ATPase (Gibbons and Rowe, 1965). Regulation of ciliary activity by cyclic nucleotides or Ca^{2+} can be explored by adding them to permeabilized cells, and observing the effects on the reactivated motility. Consistent with evidence *in vivo*, Ca^{2+} addition to permeabilized cells of *Paramecium* produces backward swimming (Naitoh and Kaneko, 1972), whereas cyclic AMP addition elicits fast forward motion (Nakaoka and Ooi, 1985; Bonini *et al.* 1986; Bonini and Nelson, 1988). Cyclic GMP also regulates *Paramecium* motility, producing fast forward swimming upon addition to models (Nakaoka and Ooi, 1985; Bonini and Nelson, 1988). However, cyclic GMP differs from cyclic AMP in the regulation of the helical swimming path of the cell, probably reflecting differential regulation of ciliary beat direction (Bonini and Nelson, 1988). Cyclic AMP and cyclic GMP are also distinct in their ability to reverse Ca^{2+}-induced backward swimming, cyclic AMP being more effective.

In the control of sperm flagellar motility, cyclic AMP activates the flagellar beat through protein kinase activation and phosphorylation of target proteins (Ishiguro *et al.* 1982; Opresko and Brokaw, 1983; Tash *et al.* 1984). The cilia of *Paramecium* contain both cyclic AMP- and cyclic GMP-dependent protein kinases at relatively high specific activities (Lewis and Nelson, 1980; Schultz and Jantzen, 1980; Mason and Nelson, 1989*a*; Miglietta and Nelson, 1988). These enzymes are probably proximal targets of cyclic nucleotide action in permeabilized cells (Greengard, 1978). The biochemistry that underlies the differential regulation of *Paramecium* motility by cyclic AMP and cyclic GMP is unknown, but may in part lie in the differential phosphoprotein substrate specificity of the respective protein kinase activities. How Ca^{2+} functions to influence ciliary and flagellar beat is not clear, but protein phosphorylation appears also to be critical to Ca^{2+} control (Segal and Luck, 1985; Tash and Means, 1982, 1987; Tash *et al.* 1988).

Here we describe the phosphorylation of cellular and ciliary proteins of permeabilized cells by endogenous protein kinase activities under the same conditions as those in which we observe a motile response to cyclic AMP, 8-bromo cyclic GMP (8-Br-cyclic GMP) and Ca^{2+}. Many phosphoprotein substrates were detectable upon cyclic AMP addition to permeabilized cells, a subset of which were targets upon 8-Br-cyclic GMP addition. Other proteins were phosphorylated when Ca^{2+} was added, including a 25K ($K=10^3 M_r$) substrate that may also be a substrate for cyclic nucleotides. Among substrates phosphorylated upon cyclic AMP addition were proteins copurifying with the 22 S and 19 S dynein ATPases. Portions of this research have been presented in abstract form (Bonini and Nelson, 1985).

Materials and methods

Preparation and motility of permeabilized cells (models)

Paramecium tetraurelia, wild-type stock 51s, was grown axenically. Cells were harvested from growth medium and permeabilized with 0.01 % Triton X-100 as described (Bonini and Nelson, 1988). Protease inhibitors (1 $\mu g\,ml^{-1}$ pepstatin A (Boehringer-Mannheim, Indianapolis, IN), 0.3 mM-phenylmethylsulfonyl fluoride, 10 $\mu g\,ml^{-1}$ leupeptin (Sigma Chemical Co., St. Louis, MO)) were included in Triton and wash solutions. For models prepared in phosphate-buffered solutions (phosphate models), 10 mM-potassium phosphate replaced the 10 mM-Tris–maleate buffer in the Triton and wash solutions.

The experimental conditions used to establish the behavioral response of permeabilized cells to cyclic nucleotides (Bonini and Nelson, 1988) were modified slightly for the detection of proteins phosphorylated in association with the physiological response of fast swimming. The ATP concentration was decreased from 4 to 1 mM to increase the specific activity of the labelled ATP. This diminished slightly the extent of stimulation of swimming by cyclic nucleotides. The cell concentration was also increased to provide more protein for biochemical analysis. This shifted the half-maximal cyclic nucleotide concentration of permeabilized cells in Tris–maleate solutions from 0.5 μM for 50 000 cells ml^{-1} (Bonini and Nelson, 1988) to about 5 μM for 500 000 cells ml^{-1}. Presumably, either the cells were degrading the added cyclic nucleotide by phosphodiesterase (PDE) activity insensitive to inhibition by isobutylmethylxanthine (IBMX), or cellular receptors for cyclic nucleotide were reducing the effective cyclic nucleotide concentration.

We also used a second type of cell preparation in which cells were permeabilized and reactivated in solutions with a 10 mM-potassium phosphate buffer (phosphate models) replacing the Tris–maleate buffer (Tris–maleate models). The *Paramecium* cyclic AMP-dependent protein kinase shows greater cyclic AMP dependence *in vitro* in phosphate than in other buffers (Mason, 1987). Unlike Tris–maleate models that are reactivated to swim in MgATP, the cilia of phosphate models are reactivated to beat very poorly in MgATP alone, but cyclic nucleotide addition results in a large increase in speed (Bonini and Nelson, 1988). Both permeabilized-cell preparations responded to cyclic nucleotide addition by swimming fast, although the dose–response curves for cyclic AMP and cyclic GMP at 50 000 cells ml^{-1} required about 5 μM for a half-maximal increase in speed for phosphate models compared to around 0.5 μM for Tris–maleate models. The maximal speed of phosphate models with cyclic GMP or 8-Br-cyclic GMP was about 80 % of that with cyclic AMP. Both types of permeabilized cell preparations exhibited similar maximal speeds with cyclic AMP.

For experiments involving the isolation of cilia from phosphate models, the concentration of cells was higher (10^6 cells ml^{-1}). This shifted the half-maximum for the swimming response to about 100 μM cyclic nucleotide. For such experiments, we added 200 μM cyclic AMP or 8-Br-cyclic GMP. Although high concentrations of cyclic nucleotide were required in these experiments, the behavioral specificity of the responses to cyclic AMP and cyclic GMP remained intact (Bonini and Nelson, 1988).

Protein phosphorylation of permeabilized cells

Protein phosphorylation was done with 5×10^4 to

1×10^6 cells ml^{-1} in 10 mM-Tris–maleate or potassium phosphate, pH 6.4, with 10 mM-EGTA, 50 mM-potassium acetate, 4 mM-$MgCl_2$, 1 mM-ATP, between 50 and 100 μCi of [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) per μmol ATP, plus other additions as noted. Cyclic nucleotides and analogs were purchased from Sigma (St Louis, MO). For Ca^{2+} solutions, $CaCl_2$ was added to obtain the desired free Ca^{2+} concentration using the dissociation constants of Ca^{2+}, Mg^{2+} and H^+ for ATP and EGTA given by Martell and Smith (1974) and Chaberek and Martell (1959). Phosphorylation was initiated by addition of cells, and experiments were performed at 20–22°C for up to 5 min. For studies of whole models, labelling was terminated by addition of 10 % trichloroacetic acid (TCA), and protein was allowed to precipitate for at least 30 min on ice prior to solubilization in sample buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

All experiments were repeated at least three times with independent cell preparations.

Subfractionation of permeabilized cells

Divalent cations such as Ca^{2+} deciliate intact cells (Adoutte *et al.* 1980). Apparently an intact membrane is not required for the effectiveness of these agents, since permeabilized cells were also deciliated by the addition of Ca^{2+} (10 mM) or Ba^{2+} (2 mM). We used Ba^{2+} to deciliate and obtain ciliary fractions because this deciliation left cell bodies intact, as judged by phase-contrast microscopy, unlike Ca^{2+}, which could rupture cell bodies. Cilia isolated after deciliation with either Ba^{2+} or Ca^{2+} were similar in SDS–PAGE protein pattern and ATPase activity that was extractable with high salt solutions.

For the isolation of ciliary proteins from reactivated, labelled, permeabilized cells, labelling with [γ-^{32}P]ATP was arrested by addition of 6 mM-ATP to dilute the specific activity of the ATP, and of 6 mM-NaF to inhibit phosphatase activity (Lewis and Nelson, 1981). $BaCl_2$ was then added to a final concentration of 2 mM to deciliate, and models were gently vortexed 30 s. Bodies were pelleted by centrifugation (4400 ***g***, 30 s, Beckman Microfuge), and the ciliary suspension was collected. Subsequent steps were performed at 4°C, and protease inhibitors (5 μg ml^{-1} pepstatin A, 0.3 mM-phenylmethylsulfonyl fluoride, 10 μg ml^{-1} leupeptin) were included in all solutions.

The ciliary suspension was separated into a ciliary pellet and deciliation supernatant by centrifugation for 10 min at 11 000 ***g*** (Beckman Microfuge) or for 15 min at 18 000 revs min^{-1} (JA-21 rotor). The deciliation supernatant was recentrifuged, and the ciliary pellet was washed once if samples were to be prepared for SDS–PAGE. For preparation of subciliary fractions, the ciliary pellet was washed and resuspended at 1 to 2 mg ml^{-1} in 1 % Triton X-100 in MMKFED (20 mM-Mops, 1 mM-$MgCl_2$, 100 mM-KCl, 10 mM-NaF, 0.01 mM-EDTA, 1 mM-dithiothreitol, pH 7.5). Cilia were vortexed gently for 10 s every 3 min for a total of 20 min, then axonemes were pelleted at 18 000 revs min^{-1} for 15 min (JA-21 rotor) or at 11 000 ***g*** (Beckman Microfuge) for 5 min. The Triton supernatant was collected and recentrifuged prior to preparation of samples for SDS–PAGE. Axonemes were washed once in MMKFED, then dyneins were extracted with 0.6 M-NaCl for 30 min (Travis and Nelson, 1988*a*). Crude dynein was separated from the extracted axonemes by centrifugation for 30 min at 30 000 revs min^{-1} (SW 50.1 rotor) or for 10 min at 100 000 ***g*** (Beckman Airfuge).

Crude dynein was concentrated if necessary by ultrafiltration in Amicon Centricon-10 microconcentrators, then 0.5 ml samples were layered on 5 % to 30 % linear sucrose gradients (12.5 ml) of 90 mM-NaCl, 10 mM-NaF, 0.1 mM-EDTA, 1 mM-dithiothreitol, 20 mM-Mops, pH 7.5, with protease inhibitors. Sucrose gradients were centrifuged for 17 h at 36 000 revs min^{-1} (SW 40Ti rotor) and fractions of 800 μl were collected from the bottom of the tube. For SDS–PAGE, samples were precipitated overnight with 0.2 mg ml^{-1} deoxycholate and 10 % TCA, then solubilized in SDS–PAGE sample buffer. Protein was measured by Bradford assay (Bradford, 1976; Bradford reagent from Pierce Chemical Co., Rockford, IL) or by the Lowry method (Lowry *et al.* 1951) following deoxycholate–TCA precipitation (Peterson, 1983), with bovine serum albumin as a standard.

SDS–PAGE and autoradiography

TCA-precipitated samples were centrifuged 1 min at 11 000 ***g*** in a Beckman Microfuge, washed with 1 ml ice-cold water to remove residual TCA, and solubilized in SDS–PAGE sample buffer (5.3 % SDS, 8.8 % β-mercaptoethanol, 17.5 % glycerol, 198 mM-Tris base, 17.5 mM-EDTA, pH 6.8). Samples were heated in a boiling water bath for 2–20 min, centrifuged at 11 000 ***g*** for 5–10 min, and loaded on gels. SDS–polyacrylamide gels were linear 5 % to 15 % gradients with 3 % or 4 % stacking gels (Laemmli, 1970). Molecular weight markers were myosin (200K), β-galactosidase (116K), phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), glyceraldehyde 3-phosphate dehydrogenase (36K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K), and lysozyme (14.4K) (BioRad, Richmond, CA; except for glyceraldehyde 3-phosphate dehydrogenase, which was purchased from Sigma). Gels were stained with silver, according to Poehling and Neuhoff (1981) or with Coomassie (0.01 % Coomassie Brilliant Blue, 45 % ethanol, 45 % acetic acid, and destained in 25 % isopropanol, 7.5 % acetic acid). Prior to drying, gels were generally incubated for several hours in 15 % methanol, 3 % glycerol.

Autoradiography was performed on gels dried under vacuum onto either cellophane for silver-stained gels or Whatman filter paper for Coomassie-stained gels. Gels were exposed to Kodak XAR-5 or XRP-5 X-ray film for 1 day to 3 weeks. When intensifying screens were used (Dupont Cronex lightening plus), film was pre-flashed to 0.1 O.D. unit and exposures were performed at −80°C (Laskey and Mills, 1977). Densitometry was performed on autoradiographs using a Zeineh Soft Laser Scanning Densitometer (model SL-504-X4, Biomed Instruments, Inc.). For quantification, relative peak heights measured from a baseline of the bottom of the scan to the top of the peak of interest were compared.

Results

Cyclic nucleotide-dependent phosphorylation in permeabilized cells

Permeabilized cells, reactivated to swim with MgATP, respond to cyclic AMP or cyclic GMP addition by increasing forward swimming speed. To determine whether the effects of cyclic AMP and cyclic GMP on ciliary motility were associated with an increase in the phosphorylation of specific target proteins, permeabilized cells were reactivated to swim in the presence of Mg[γ-^{32}P]ATP in the presence or absence of cyclic nucleotide. Phosphoproteins labelled by endogenous protein kinase activities in association with the motile response were analyzed by SDS–PAGE and autoradiography (Fig. 1; Table 1). The motile response to cyclic AMP and cyclic GMP occurs within 30 s (Bonini and Nelson, 1988); therefore, proteins whose phosphorylation is relevant to the response should be labelled to

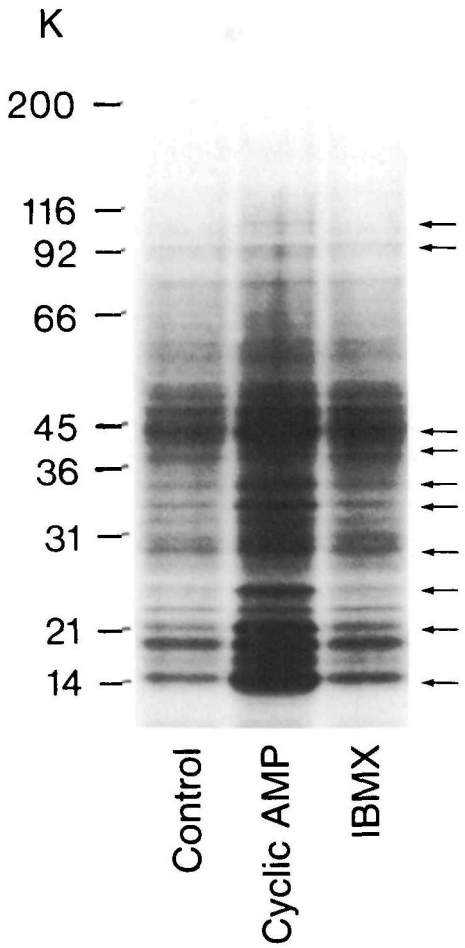

Fig. 1. Effects of cyclic AMP and IBMX on protein phosphorylation in permeabilized cells. Permeabilized cells were labelled for 2 min in Tris–maleate buffer at 200 000 cells ml^{-1} in the presence of [γ-^{32}P]ATP alone (control) or plus 100 μM-cyclic AMP, or 1 mM-IBMX, and protein phosphorylation was analyzed by SDS–PAGE (200 μg/lane) followed by autoradiography. The autoradiograph is presented. Cells incubated with 100 μM 5′-AMP, 5′-GMP, adenosine or NaCl had phosphorylation patterns identical to the control or IBMX conditions. None of these compounds, other than cyclic AMP, increases the swimming speed of permeabilized cells (Bonini and Nelson, 1988). Arrows on the right indicate the substrates, from bottom to top, of 15, 22, 25, 28, 33, 35, 39, 44, 95 and 110K.

functionally significant levels by this time. In preliminary experiments we found that the same proteins were labelled at 30 s, 2 min and 5 min, so we typically incubated with labelled ATP for 2 min to allow sufficient incorporation of ^{32}P for easier detection of substrates by autoradiography. The proteins of whole models labelled upon cyclic nucleotide addition, and the extent of phosphorylation after 2 min, were similar for permeabilized cells prepared in Tris–maleate and in phosphate buffers. Digestion of samples with protease K prior to gel electrophoresis caused the loss of all labelled bands in the

Table 1. *Substrate phosphorylation with cyclic AMP, 8-Br-cyclic GMP and Ca^{2+} in permeabilized cells*

Substrate relative molecular mass (K)	Fold increase in phosphorylation over control (mean±S.D., n=5–16)		
	Cyclic AMP	8-Br-cyclic GMP	Ca^{2+}
14	n.d.	n.d.	5±3
15	2.7±0.4	1.7±0.2	n.d.
22	2.3±0.5	1.2±0.1	n.d.
25	2.2±0.4	1.2±0.1	1.5±0.2
28	1.5±0.2	1.3±0.1	n.d.
33	2.2±0.3	1.1±0.1	n.d.
35	1.8±0.3	1.4±0.5	n.d.
44	1.7±0.3	1.1±0.1	n.d.
58	n.d.	n.d.	1.7±0.2
95	1.5±0.3	1.4±0.1	n.d.
110	1.8±0.3	1.6±0.2	n.d.

Autoradiographs of whole model protein labelled for 2 min at 40 000–300 000 cells ml^{-1} with between 10 and 100 μM cyclic nucleotide or 1.5 μM-Ca^{2+} were scanned with a densitometer. Peak heights from a baseline at the bottom of the scan were measured. Maximal phosphorylation is presented as fold increase±S.D. of the control peak height (n=number of independent experiments). Data for Tris–maleate and phosphate models did not differ for these substrates within the standard deviation, and are combined. The 39K substrate was not detectable by densitometry. n.d., not determined (did not appear to be phosphorylated above control levels).

autoradiographs (not shown), indicating that ^{32}P was being incorporated into protein, not nucleic acid.

Although the background level of phosphorylation in permeabilized cells in the absence of cyclic nucleotide was high, we were able to distinguish reproducible substrates of cyclic AMP- and 8-Br-cyclic GMP-activated protein kinase activity (Fig. 1; Table 1). Cyclic AMP addition to permeabilized cells stimulated the phosphorylation of 10 proteins, of 15, 22, 25, 28, 33, 35, 39, 44, 95 and 110K. Proteins of 120, 175 and >300K were also substrates, but were sometimes difficult to detect because the label was faint.

A subset of the cyclic AMP-stimulated phosphoproteins also served as substrates for kinase activity stimulated by the addition of 8-Br-cyclic GMP, a selective activator of the cyclic GMP-dependent protein kinase of *Paramecium* (Miglietta and Nelson, 1988). Some substrates were phosphorylated to similar levels by 8-Br-cyclic GMP and cyclic AMP (28, 39, 95, 110K); some were not significantly phosphorylated by 8-Br-cyclic GMP (33 and 44K); and others had reduced levels of phosphorylation compared to cyclic AMP (15, 22, 25 and 35K; Table 1). When detectable, the 120 and 175K substrates were phosphorylated by 8-Br-cyclic GMP- and cyclic AMP-stimulated protein kinase activities; both nucleotides had about the same effect.

The concentration of cyclic nucleotide required for half-maximal phosphorylation, determined by densitometric scanning of the phosphorylation of the 15K substrate, was 0.36±0.03 μM (mean±S.D., n=5) for Tris–maleate models and 0.7±0.6 μM (mean±S.D., n=8) for phosphate models (cyclic AMP and 8-Br-cyclic GMP data were similar and are combined). Increasing the cyclic nucleotide concentration to 100 μM for Tris–

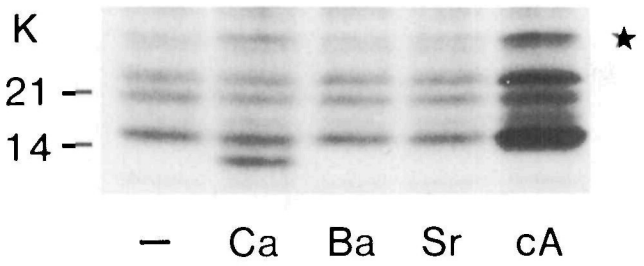

Fig. 2. Effects of Ca^{2+}, Ba^{2+}, Sr^{2+} and cyclic AMP on protein phosphorylation in permeabilized cells. Cells swam backward upon addition of Ca^{2+}, Ba^{2+} or Sr^{2+}; however, Ba^{2+} and Sr^{2+} did not stimulate detectable phosphorylation. Phosphate models were reactivated for 2 min at 200 000 cells ml^{-1} in Mg[γ-^{32}P]ATP alone or with 1.5 μM-Ca^{2+}, 100 μM-Ba^{2+} or 25 μM-Sr^{2+} (free concentrations) or with 100 μM cyclic AMP. Protein phosphorylation was analyzed by SDS–PAGE (350 μg/lane) followed by autoradiography. The star indicates the 25K substrate of Ca^{2+} and cyclic nucleotides.

maleate models and 200 μM for phosphate models resulted in phosphorylation patterns similar to those at lower cyclic nucleotide concentrations; no new phosphoproteins appeared. Addition of adenosine, 5′-AMP, 5′-GMP (not shown) or IBMX (Fig. 1) to MgATP-reactivated cells did not stimulate protein phosphorylation over levels seen with MgATP alone. These compounds also do not stimulate the motility of permeabilized cells (Bonini and Nelson, 1988). The phosphorylation of proteins in Tris–maleate models was reversed within 30 min after removal of the radioactive ATP (not shown), indicating the presence of phosphoprotein phosphatase activity in the permeabilized cell preparation.

Ca^{2+}-dependent protein phosphorylation in permeabilized cells

Addition of Ca^{2+} to permeabilized cells induces backward swimming and antagonizes the abilities of cyclic AMP and cyclic GMP to stimulate forward swimming speed (Nakaoka and Ooi, 1985; Bonini and Nelson, 1988). The mechanism by which Ca^{2+} functions to reverse ciliary beat, or by which cyclic nucleotides and Ca^{2+} interact biochemically in cells is not known. We explored the possibility that Ca^{2+} may also induce protein phosphorylation, and that cyclic nucleotides and Ca^{2+} may have some common substrates.

In whole models, Ca^{2+} consistently induced the phosphorylation of four major substrates (14, 25, 58 and 155K; Fig. 2, Table 1, and data not shown). Detectable phosphorylation occurred by 0.5 μM, a concentration that coincides with the onset of a behavioral response to Ca^{2+} (Nakaoka and Kaneko, 1972; Bonini and Nelson, 1988). Maximal phosphorylation was obtained with 1 to 5 μM-free Ca^{2+}. The 25K Ca^{2+} substrate had the same mobility on SDS–PAGE as the 25K substrate of cyclic AMP and 8-Br-cyclic GMP (Fig. 2; Table 1), and thus represents a potential site of integration of these second messengers.

Ba^{2+} and Sr^{2+} are Ca^{2+} analogs, which at 100 μM-Ba^{2+} and 25 μM-Sr^{2+} (free concentrations) caused a backward swimming response of permeabilized cells similar to that of Ca^{2+} (Naitoh and Kaneko, 1973; not shown). However, neither Ba^{2+} nor Sr^{2+} at these concentrations stimulated detectable protein phosphorylation in whole models (Fig. 2). The apparent inability of Ba^{2+} and Sr^{2+} to stimulate Ca^{2+}-dependent protein kinase activity is consistent with the properties of the characterized Ca^{2+}-dependent protein kinase of *Paramecium* (Gundersen and Nelson, 1987; Son and Nelson, unpublished observations). The Ca^{2+}-stimulated phosphorylation of the substrates we detect in whole models appears, therefore, not to be obligatory for reversal of ciliary beat direction.

Antagonism between Ca^{2+}-induced ciliary reversal and cyclic AMP- and cyclic GMP-induced fast forward swimming (Bonini and Nelson, 1988) is also seen with backward swimming induced by Ba^{2+} or Sr^{2+} addition (not shown). The mechanism that underlies the antagonism with cyclic nucleotides must therefore be sensitive to Ba^{2+} and Sr^{2+}, as well as to Ca^{2+}. These results suggest that a mechanism other than Ca^{2+}-induced phosphorylation may be involved.

Cyclic nucleotide-stimulated phosphoproteins of cilia and deciliation supernatant from permeabilized cells

Proteins that are regulated by cyclic nucleotide-dependent protein phosphorylation and that function in the behavioral responses to cyclic AMP and cyclic GMP may be localized to cilia. A protocol was therefore developed to deciliate permeabilized cells following reactivation of motility. This enabled an analysis to be made of phosphoproteins of the ciliary compartment that were labelled in association with the motile response of whole models to cyclic nucleotides. Not all ciliary proteins are found in these detached cilia. Upon deciliation, cilia break at the base between the necklace and plaque regions (Blum, 1971; Satir *et al.* 1976; Adoutte *et al.* 1980). Some structures within the transition zone (Dute and Kung, 1978; Hoops and Witman, 1985), and possibly the basal body and associated fibers within the cell body, are left behind in deciliated bodies. However, in order to examine potential targets located along the length of the axoneme, such as dynein, we studied isolated cilia from labelled, reactivated cells.

The ciliary suspension contained approximately 8 % of the total protein of permeabilized cells (780±140 μg, mean±S.D., n=11, from 1×10^6 cells). This fraction consisted of cilia (27 %±5 %, mean±S.D., n=9, of the total protein of the ciliary suspension), as well as of soluble proteins that comprised the deciliation supernatant. The majority of the protein of the ciliary suspension was in the deciliation supernatant (83 %±20 %, mean±S.D., n=10), and the protein and phosphorylation patterns of the ciliary suspension and deciliation supernatant were similar (data not shown). A comparison of the ^{32}P incorporated per total μg protein of permeabilized cells, deciliation supernatant and ciliary pellet indicated that specific radioactivity in the deciliation supernatant was about ten times that of permeabilized cells, and in the ciliary pellet was about two to four times that of permeabilized cells. This suggests that, in permeabilized

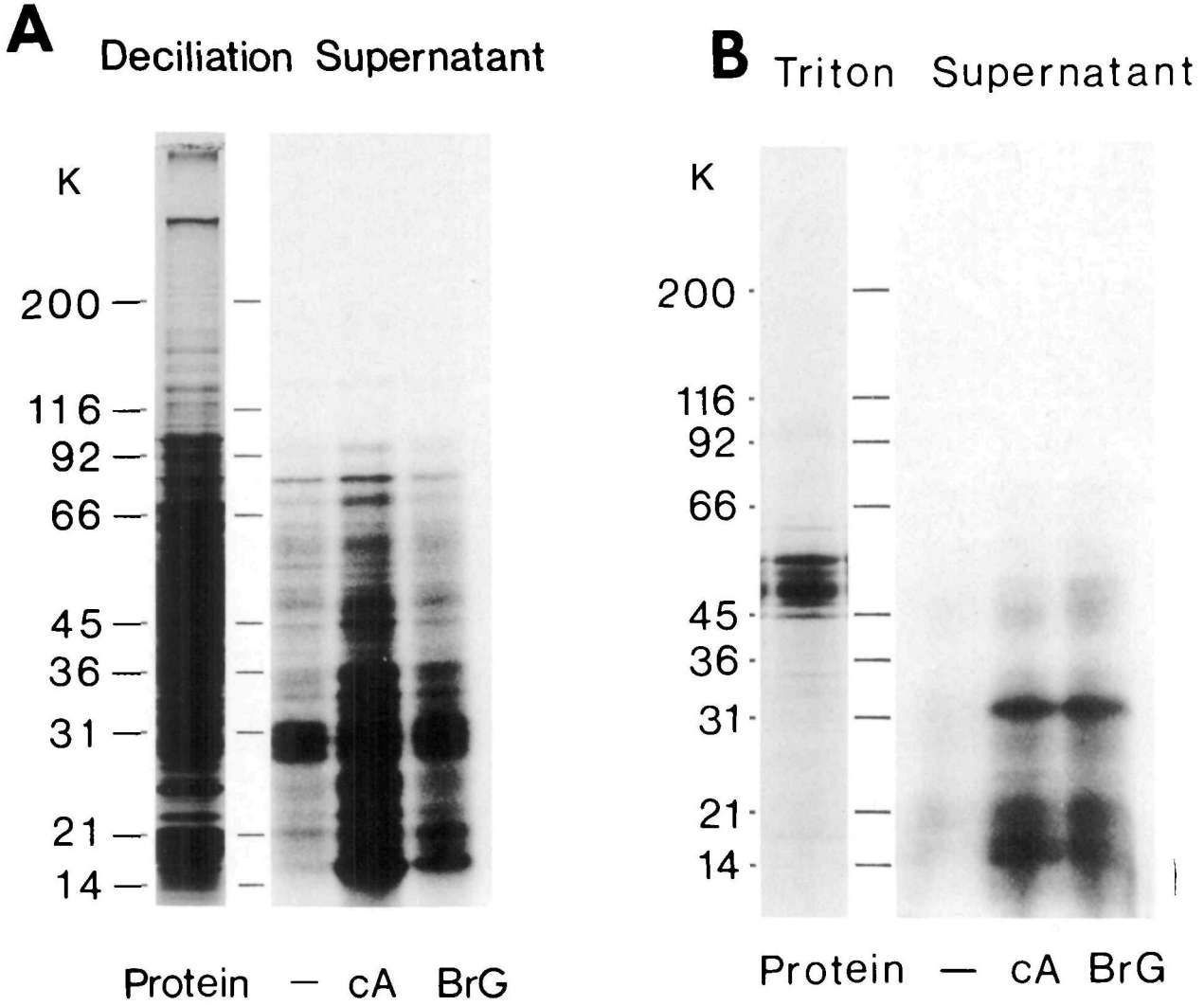

Fig. 3. Cyclic nuceotide-dependent protein phosphorylation of the deciliation supernatant (A) and the Triton supernatant (B) from labelled permeabilized cells. Permeabilized cells (1×10^6 cells ml^{-1}) were incubated for 5 min with [γ-^{32}P]ATP in phosphate-buffered solutions alone (−) or with 200 μM cyclic AMP (cA) or 8-Br-cyclic GMP (BrG). Protein phosphorylation was then arrested and permeabilized cells were deciliated by Ba^{2+} addition. The deciliation supernatant (A; 100 μg/lane) and Triton supernatant (B; 20 μg/lane) were analyzed by SDS–PAGE followed by autoradiography. The Coomassie-stained protein pattern is indicated in the left lane of each panel.

cells, selective phosphorylation of proteins that comprise the ciliary suspension may have occurred.

Several proteins whose phosphorylation was stimulated by cyclic AMP were found in the deciliation supernatant. These included substrates with molecular weights similar to those labelled in models (15, 22, 25 (31 was sometimes present), 33, 35, 44, and 95K), as well as some not detected in whole models (Fig. 3A). The 95K substrate of the deciliation supernatant was typically phosphorylated to similar extents with cyclic AMP and 8-Br-cyclic GMP, whereas the other substrates were phosphorylated more heavily in cyclic AMP than in 8-Br-cyclic GMP. Increases in phosphorylation ranged to 1.8-fold for 8-Br-cyclic GMP addition, and to 2.9-fold for cyclic AMP addition. Two phosphoproteins (52 and 72K) appeared to be cyclic AMP-specific.

Axonemes isolated from permeabilized cells were enriched in tubulin (identified by molecular weight and abundance in axonemes) and high molecular weight protein (>300K) characteristic of the ciliary dyneins (Fig. 4). Axonemal phosphoproteins stimulated by cyclic AMP addition to permeabilized cells included proteins of molecular weight similar to those labelled in autoradiographs of whole models (15, 22, 25, 33, 35 and 44K). Also labelled were eight proteins between 20 and 170K and one protein in the dynein heavy chain region. Some substrates (82, 125 and 140K) were labelled about equally upon cyclic AMP and 8-Br-cyclic GMP addition to permeabilized cells. Phosphorylation of the other substrates was less with 8-Br-cyclic GMP than with cyclic AMP. Cyclic AMP-specific targets of the cilia included the 25 and 170K substrates and the protein in the dynein heavy chain region. Increases in phosphorylation of axonemal substrates with cyclic AMP were 7(±1)-fold

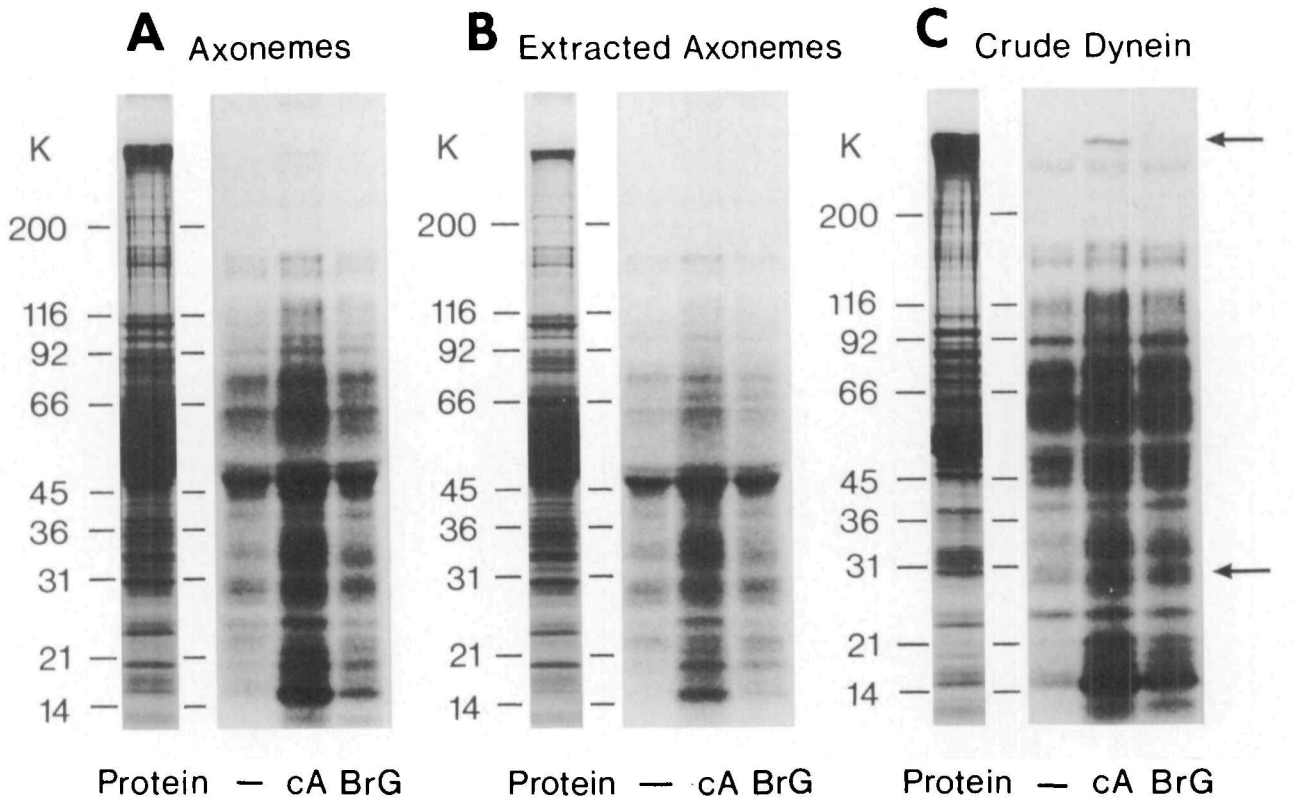

Fig. 4. Cyclic nucleotide-dependent protein phosphorylation of axonemes, crude dynein and extracted axonemes from cilia of permeabilized cells. Phosphate models (1×10^6 cells ml^{-1}) were labelled for 5 min with [γ-^{32}P]ATP alone (−) or with 200 μM-cyclic AMP (cA) or 8-Br-cyclic GMP (BrG). Phosphorylation was then arrested and permeabilized cells were deciliated. Axonemes (A) were extracted with 0.6 M-NaCl to yield a high-speed supernatant of crude dynein (C), and pellet of extracted axonemes (B). Samples of 100 μg per lane were analyzed by SDS–PAGE followed by autoradiography. The Coomassie-stained protein pattern of each respective fraction is included in the left lane of each panel. Arrows on the far right indicate the positions of the high molecular weight (top arrow) and 29K (bottom arrow) substrates of cyclic AMP addition that were extracted by high salt treatment into the crude dynein supernatant.

(mean±S.D.; $n=3$) for the 15K protein, and between 1.5- and 4-fold for other substrates. Phosphorylation with 8-Br-cyclic GMP addition was stimulated 3.2(±0.7)-fold ($n=3$) for the 15K substrate, and from 1.5- to 2.6-fold above control for other substrates. The treatment with 1% Triton X-100 to obtain demembranated axonemes preferentially extracted a 33K ciliary substrate (Fig. 3B).

The phosphoproteins of 44 and 47K may be forms of the regulatory subunits of the cyclic AMP-dependent protein kinases of *Paramecium*. Proteins of similar molecular weight in permeabilized cells, deciliation supernatant and cilia isolated from permeabilized cells cross-reacted with monoclonal antibodies raised against the regulatory subunits of the cyclic AMP-dependent protein kinase (data not shown; see Bonini, 1987; Hochstrasser, 1989; Hochstrasser and Nelson, 1989). Immunological evidence also showed that the catalytic subunit of the cyclic AMP-dependent protein kinase was present in permeabilized cells. The presence and distribution of the regulatory and catalytic subunits of the cyclic AMP-dependent protein kinase in the subfractions from models, compared with whole cells (Mason and Nelson, 1989*a*), suggests that the models are not grossly disrupted with respect to kinase localization.

Cyclic AMP-stimulated phosphorylation of dynein-associated proteins

Dynein activity is released from demembranated axonemes by treatment with high salt solution (Gibbons, 1963). To determine whether any of the proteins phosphorylated upon stimulation of permeabilized cells with cyclic AMP or 8-Br-cyclic GMP were associated with dynein, the axonemes from labelled permeabilized cells were extracted with high salt solution, yielding a crude dynein supernatant (approximately 35% of total axonemal protein) and an extracted axoneme pellet. Crude dynein was enriched in high molecular weight proteins (dynein heavy chains), whereas the majority of the tubulin remained in the pellet. Most of the cyclic AMP- and 8-Br-cyclic GMP-stimulated phosphoproteins of axonemes were extracted by high salt solution, becoming enriched in specific activity in the crude dynein fraction (compare Fig. 4B and 4C). In particular, the cyclic AMP-specific phosphoprotein of >300K was extracted by high salt solution, consistent with its being a dynein heavy chain. The phosphorylation of this protein was stimulated 4(±2)-fold ($n=5$) above the control. Cyclic AMP substrates of 25 and 35K remained with the extracted axoneme pellet.

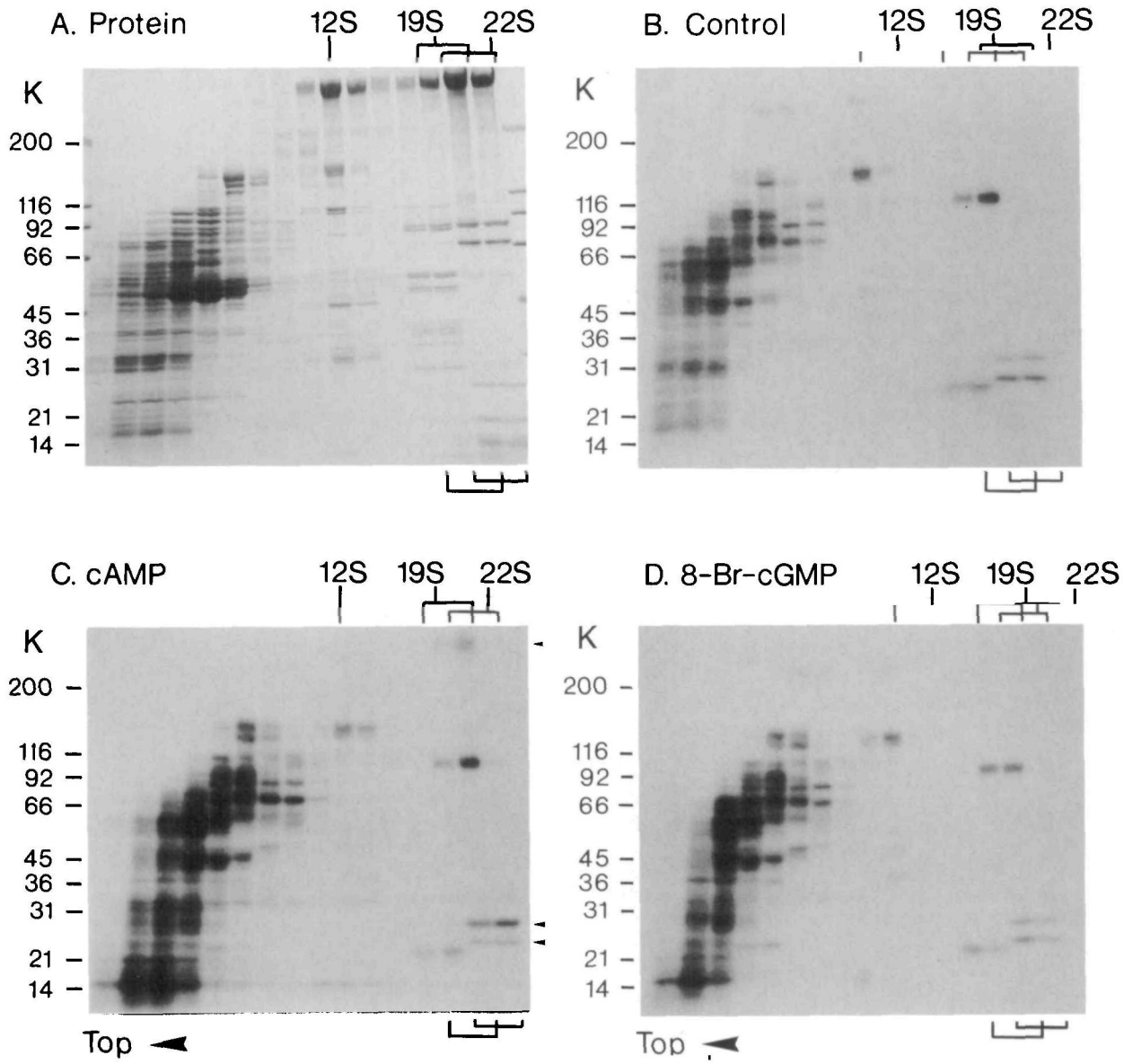

Fig. 5. Cyclic nucleotide-dependent protein phosphorylation of sucrose gradient-purified dyneins from cilia of labelled permeabilized cells. Phosphate models were reactivated at 1×10^6 cells ml^{-1} for 5 min in the presence of [γ-^{32}P]ATP alone, or with 200 μM cyclic AMP or 8-Br-cyclic GMP. Phosphorylation was then arrested and cilia removed by deciliation with Ba^{2+}. Ciliary dyneins were extracted by 0.6 M-NaCl and separated by sucrose density gradient centrifugation. Fractions were collected and analyzed by SDS–PAGE followed by autoradiography. Fractions from the gradient below the position of 22 S dynein were not included on the gel, since they contained no detectable proteins or phosphoproteins. A. Protein: Coomassie-stained protein pattern of the fractions from the sucrose gradient corresponding to the autoradiograph of the control. Positions of the 22 S and 12 S dynein ATPases are identified by the molecular weight of the proteins characteristic of these peak fractions of ATPase activity. The major polypeptides of 22 S dynein (>300K, 81K, 64K, 21K, 16K, and several of 12–14K; Travis and Nelson, 1988*a*) are in the two rightmost lanes. The 19 S ATPase is a shoulder of the 22 S dynein (Travis and Nelson, 1988*a*).
B. Control: phosphorylation pattern of dyneins from cilia of permeabilized cells reactivated in MgATP alone. C. Cyclic AMP: phosphorylation pattern of dyneins from cilia of cells reactivated in MgATP plus cyclic AMP. Arrowheads on right indicate (from the top) the high molecular weight phosphoprotein in the 19 S region, and the 29K and 25K proteins associated with 22 S dynein. D. 8-Br-cyclic GMP: phosphorylation pattern of dyneins from cilia of cells reactivated in MgATP plus 8-Br-cyclic GMP.

Crude *Paramecium* ciliary dynein contains two major dynein ATPase activities, which sediment on sucrose gradients at 22 S and 12 S (Travis and Nelson, 1988*a*). In addition, a minor dynein sediments at 19 S as a shoulder of 22 S dynein. The positions of the ATPases can be identified by the characteristic protein patterns of heavy, intermediate and light molecular weight chains on SDS–PAGE (Travis and Nelson, 1988*a*; Fig. 5A). Proteins that sedimented with the 22 S and 19 S dyneins were targets of cyclic AMP-stimulated protein phosphoryl-

ation in permeabilized cells (Fig. 5B and 5C). Two proteins in the 22 S region were phosphorylated in permeabilized cells (29 and 25K). The phosphorylation of the 29K protein was stimulated upon addition of cyclic AMP to permeabilized cells. Phosphorylation of the 25K protein appeared to be inhibited by cyclic AMP. The 19 S dynein region had a major phosphoprotein at 110K that did not appear to be stimulated by cyclic nucleotide addition. A cyclic AMP-specific phosphoprotein of high molecular weight (>300K) sedimented in the 19 S region (Fig. 5C). The 12 S dynein ATPase contained one major phosphoprotein at 160K that did not appear to be stimulated upon cyclic nucleotide addition to models. The 8-Br-cyclic GMP- (Fig. 5D) and other cyclic AMP-stimulated phosphoproteins of the crude dynein fraction sedimented in sucrose gradients at less than 12 S. Most of the ^{32}P-labelled proteins of the high salt extract were in these fractions.

Discussion

We report that cyclic AMP stimulation of ciliary motility in *Paramecium* is accompanied by the phosphorylation of several proteins of permeabilized cells and subciliary fractions, including two proteins copurifying with the ciliary dyneins. 8-Br-cyclic GMP, a specific activator of the cyclic GMP-dependent protein kinase (Miglietta and Nelson, 1988), stimulated motility and the phosphorylation of a subset of the cyclic AMP-stimulated phosphoproteins, which did not include the dyneins. Cyclic AMP and 8-Br-cyclic GMP have nonidentical functions in the control of axonemal motility (Bonini and Nelson, 1988); these data suggest that differential phosphorylation upon cyclic AMP and 8-Br-cyclic GMP addition to models may in part contribute to their distinct effects.

Accumulating evidence indicates that many ciliated and flagellated cells use cyclic AMP as a second messenger for motility control (Garbers and Kopf, 1980; Tash and Means, 1983; Satir, 1985; Brokaw, 1987). In *Paramecium*, cyclic AMP couples membrane hyperpolarization to the axonemal response of fast forward swimming (Hennessey *et al.* 1985; Nakaoka and Ooi, 1985; Bonini *et al.* 1986; Bonini and Nelson, 1988). Cyclic GMP also functions as a second messenger to regulate *Paramecium* motility. *In vivo*, cyclic GMP levels increase upon prolonged membrane depolarization that is associated with fast backward swimming or with adaptation of ciliary beat direction for the resumption of forward motion (Majima *et al.* 1986; Schultz *et al.* 1986). In permeabilized cells, cyclic GMP causes fast forward swimming similar to cyclic AMP (Nakaoka and Ooi, 1985; Bonini and Nelson, 1988), but differs strikingly in its effect on the helical swimming path of the cell and in interactions with Ca^{2+} (Bonini and Nelson, 1988). Although the physiological role of cyclic GMP is not yet clear, these data suggest distinct functions of cyclic AMP and cyclic GMP in the regulation of axonemal motility.

The responses to cyclic AMP and cyclic GMP may be mediated through activation of their respective cyclic nucleotide-dependent protein kinase activities, and subsequent phosphorylation of proteins that modulate ciliary motion (Greengard, 1978). Cyclic AMP-dependent phosphorylation appears central to the control of ciliary and flagellar beat (Garbers and Kopf, 1980; Tash and Means, 1983; Satir, 1985; Brokaw, 1987). *Paramecium* ciliary motility is one of few known examples of cyclic GMP-modulated processes (Goldberg and Haddox, 1977; Lincoln and Corbin, 1983). Our results suggest that protein phosphorylation accompanies motility regulation by both cyclic AMP and cyclic GMP. *Paramecium* cilia are enriched in cyclic AMP- and cyclic GMP-dependent protein kinase activities, and much of the cyclic AMP-dependent activity is tightly bound to the axoneme, suggesting a specific function in motility (Miglietta and Nelson, 1988; Mason and Nelson, 1989*b*). Many phosphoprotein substrates of endogenous cyclic nucleotide-dependent protein kinase are present in *Paramecium* cilia (Lewis and Nelson, 1980; Eistetter *et al.* 1983; Hamasaki *et al.* 1989), including proteins copurifying with the dynein ATPases (Travis and Nelson, 1988*b*).

To identify proteins whose phosphorylation correlates with the motile responses to cyclic AMP, cyclic GMP and Ca^{2+}, we characterized phosphoproteins labelled with ^{32}P in permeabilized cells with the physiological response of motility intact. The Ca^{2+} concentrations required to initiate ciliary reversal (Naitoh and Kaneko, 1972; Bonini and Nelson, 1988) and to activate the Ca^{2+}-dependent protein kinases (Gundersen and Nelson, 1987) are similar, although no function for Ca^{2+}-stimulated phosphorylation in ciliary regulation emerged from our studies. The Ca^{2+} analogs Ba^{2+} and Sr^{2+} did not stimulate detectable protein phosphorylation, although both cause backward swimming of permeabilized cells (Naitoh and Kaneko, 1973). Other data suggest that at least some functions of Ca^{2+} may be mediated through Ca^{2+} inhibition of phosphorylation or Ca^{2+}-dependent dephosphorylation (Tash and Means, 1982, 1987; Izumi and Nakaoka, 1987; Tash *et al.* 1988; Hamasaki *et al.* 1989). Interplays between phosphorylation and dephosphorylation may be particularly relevant to antagonism between cyclic nucleotides and Ca^{2+}.

Proteins phosphorylated in whole models upon cyclic nucleotide addition included proteins released into the ciliary suspension upon deciliation. These consisted of both soluble proteins of the deciliation supernatant, as well as proteins associated with the ciliary pellet. Proteins of the deciliation supernatant from permeabilized cells, enriched in many cyclic AMP-dependent phosphoproteins, may have originated from the intraciliary compartment (Riddle *et al.* 1982), ciliary or cell membrane, and/or the cell cytoplasm. Eistetter *et al.* (1983) showed the phosphorylation in isolated cilia of proteins of 41, 43, 53 and 65K. The cyclic AMP-specific 52 and 72K substrates that we detect in the deciliation supernatant from models may correspond to these substrates. Eistetter *et al.* also found a 31K phosphoprotein in ciliary membrane vesicles. We find a phosphoprotein of similar M_r in the Triton extract of cilia from models labelled in cyclic AMP. In addition, some of these phosphoproteins may represent subunits of the cyclic AMP- and cyclic GMP-dependent protein kinases of *Paramecium* (44 or

48K: Mason and Nelson, 1989*a*,*b*; Hochstrasser and Nelson, 1989; and 77K: Miglietta and Nelson, 1988, respectively). Phosphorylation of the regulatory subunit of the cyclic AMP-dependent protein kinase is of potential significance to the control of motility, since the regulatory subunit may have functions in addition to binding the catalytic subunit, including binding to microtubule-associated proteins (Lohmann *et al.* 1984; Constantinou *et al.* 1985; DeCamilli *et al.* 1986).

The majority of the ciliary targets of cyclic AMP and 8-Br-cyclic GMP kinase activity were released by treatment with high salt solution, which also releases the axonemal dyneins. The major dyneins released upon salt treatment of *Paramecium* axonemes sediment at 22 S, 19 S and 12 S (Travis and Nelson, 1988*a*). Cyclic AMP addition to permeabilized, MgATP-reactivated cells increased the phosphorylation of a 29K protein cosedimenting with 22 S dynein, and of a high molecular weight protein in the 19 S region. The 29K protein may be either a subunit of the 22 S dynein, a copurifying regulatory element, or associated with another large structure that sediments with 22 S dynein. A protein of similar molecular weight is phosphorylated *in vitro* by cyclic AMP addition to axonemes isolated from whole cells (Eistetter *et al.* 1983; Hamasaki *et al.* 1989) and from permeabilized cells (Hamasaki *et al.* 1989). Hamasaki and Satir (1988) also report the association of the phosphoprotein with 22 S dynein, as well as potential effects on ATPase activity. Together, these data suggest the 29K cyclic AMP-stimulated phosphoprotein associated with 22 S dynein is involved in the cyclic AMP motility response. The high molecular weight protein phosphorylated in models that sedimented in the 19 S region is also a substrate *in vitro* of cyclic nucleotide-dependent protein kinase activity in cilia (Travis and Nelson, 1988*a*). The 19 S ATPase sediments with calmodulin-like activity in sucrose gradients (Travis and Nelson, 1988*b*), and thus may represent an integration site of the Ca^{2+} and cyclic AMP pathways. Whether the 19 S ATPase is a distinct dynein, or a form of the 22 S dynein is not yet clear. However, its phosphorylation both in permeabilized cells that are responding to cyclic AMP by increased motility, and *in vitro* upon cyclic AMP addition to cilia, would be consistent with potential relevance in transduction of the cyclic AMP response.

In sperm, high molecular weight proteins, which may also be dynein subunits, are phosphorylated in association with cyclic AMP stimulation of motility (Tash and Means, 1982; Opresko and Brokaw, 1983). Subunits of the *Chlamydomonas* inner and outer arm dyneins, including heavy chains and lower molecular weight components, are labelled by ^{32}P *in vivo* (Piperno and Luck, 1981, 1982), which may represent regulatory or cyclic nucleotide-dependent phosphorylation. We also found that many cyclic AMP- and 8-Br-cyclic GMP-stimulated phosphoproteins of the high salt extract sedimented with low sedimentation coefficients in sucrose gradients. These phosphoproteins may be associated with the dynein arms *in situ*, or may be parts of other axonemal structures. Although protein kinase substrates may include dynein subunits that directly regulate dynein arm activity, regulation of ciliary motion by cyclic AMP and 8-Br-cyclic GMP could also be achieved through the phosphorylation of other proteins, soluble or bound to the axoneme, or localized to the structural specializations of the ciliary base.

There is as yet no direct evidence that cyclic AMP and cyclic GMP act through protein kinase to regulate motility (Nakaoka and Ooi, 1985; Hamasaki *et al.* 1989; N.M. Bonini, unpublished observations). These studies are difficult due to a lack of specific inhibitors of the *Paramecium* enzymes (Mason and Nelson, 1989*b*; Miglietta and Nelson, 1988). The availability of purified dyneins (Travis and Nelson, 1988*a*,*b*) and cyclic AMP-dependent protein kinases (Hochstrasser, 1989) from *Paramecium* will allow us to determine whether dynein is the direct target of phosphorylation, and whether it is regulated by phosphorylation.

Other motile systems also display dynamic regulation by cyclic AMP and/or Ca^{2+} (Adelstein and Eisenberg, 1980; Johnson, 1985; Gilson *et al.* 1986; Lynch *et al.* 1986; Rozdzial and Haimo, 1986). Ciliary dynein has recently been shown to be closely related to cytoplasmic ATPases that may function in organelle and vesicle transport, or cell mitosis (Gibbons, 1987; Lye *et al.* 1987; Paschal *et al.* 1987; Vallee *et al.* 1988). Studies of ciliary regulation, therefore, may be relevant to second messenger control of these other types of cellular motility.

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